Impact of dietary protein content on uncoupling protein mRNA abundance in swine☆

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Abstract

The present study was designed to determine if dietary protein can alter uncoupling protein (UCP) expression in swine, as has been shown in rats, and attempt to identify the mechanism. Eight pigs (~50 kg body mass) were fed an 18% crude protein (CP) diet while another eight pigs were switched to a diet containing 12% crude protein (CP) and fed these diets until 110 kg body mass. The outer (OSQ) and middle (MSQ) subcutaneous adipose tissues, liver, leaf fat, longissimus (LM), red portion of the semitendinosus (STR) and the white portion of the ST (STW) were analyzed for gene expression by real-time PCR. Feeding of 12% CP did not alter growth or carcass composition, relative to 18% CP ($P > 0.05$). Serum growth hormone, non-esterified fatty acids, triglycerides and urea nitrogen were reduced with the feeding of 12% CP ($P < 0.05$). The UCP2 mRNA abundance was reduced in LM, STR, MSQ and OSQ with feeding of 12% CP ($P < 0.05$), as was UCP3 mRNA abundance in MSQ and STW ($P < 0.01$). Peroxisome proliferation activated receptor α (PPARα) and PPARγ were reduced in MSQ and STR ($P < 0.05$) with feeding 12% CP as was the PPARα regulated protein, acyl CoA oxidase (ACOX, $P < 0.05$). These data suggest that feeding 12% CP relative to 18% CP reduces serum NEFA, which reduces PPARα and PPARγ expression and consequently reduces UCP2 lipoperoxidation in OSQ and STR and also reduced UCP3 associated fatty acid transport in MSQ and STW.
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Keywords: UCP2; UCP3; PPARα; PPARγ; Lipid metabolism

1. Introduction

Dietary composition has been demonstrated to alter body composition and energy balance (Rolland-Cachera et al., 1997). The usual suspects in this metabolic shift have been dietary fat and carbohydrate. However, recent studies have implicated a role for dietary protein in this phenomenon in rodents (Dulloo and Samec, 2001; Masanés et al., 2002; Petzke et al., 2005, 2007) and swine (Adeola and Young, 1989; Cromwell et al., 1993; Gomez et al., 2002; Kerr et al., 1995). The shift in dietary protein content has been associated with changes in energy expenditure in rats (Dulloo and Samec, 2001; Petzke et al., 2007), which led to the postulate that the uncoupling proteins (UCP) may be involved in the adaptation to changes in energy expenditure with dietary protein intake. However, these studies have been contradictory. For example, feeding a low protein diet to rats results in an increase in energy expenditure and a reduction in UCP3 mRNA abundance (Dulloo and Samec, 2001); while feeding a high protein diet (>50% metabolizable energy) also depressed skeletal muscle UCP3 gene expression (Petzke et al., 2005). This contradiction is confounded by differences in feeding duration and diet composition. Irrespective of the contradiction, these studies do indicate that dietary protein can affect UCP expression.

The potential role of the uncoupling proteins in energy expenditure has recently been questioned (Esteves and Brand, 2005; Krauss et al., 2005; Ricquier, 2005). The specific roles of UCP2 and UCP3 may be more related to metabolism of reactive oxygen species (Negre-Salvayre et al., 1997; Jezek et al., 2004; MacLellan et al., 2005) and fatty acids (Svensson et

al., 1998; Hunt et al., 1999; Himms-Hagen and Harper, 2001; Jaburek et al., 2004); although a role in energy expenditure cannot be completely excluded (Fleury et al., 1997; Jezek and Garlid, 1998; Cline et al., 2001). Varying the protein concentration of the diet has been associated with changes in serum non-esterified fatty acids (Atinmo et al., 1978; Matthews et al., 1998; Gomez et al., 2002; Petzke et al., 2005, 2007), which could contribute to potential changes in UCP2 and UCP3 expression due to their functions in fatty acid metabolism.

These putative roles for UCP2 and UCP3 in fatty acid metabolism are believed the consequence of fatty acid interaction with peroxisome proliferator activated receptor (PPAR) elements within the promoter regions of these genes (Acin et al., 1999; Lentes et al., 1999). The PPARs are believed to directly interact with fatty acids to alter gene expression activity (Kliewer et al., 1997). For example, fatty acid activation of PPARα also promotes acyl CoA oxidase transcription (Tugwood et al., 1992), an enzyme that functions in peroxisomal fatty acid oxidation (Vasquez et al., 2001). Uncoupling protein 2 is also believed to function in peroxisomal fatty acid oxidation (Jezek et al., 2004). Thus, the present study was designed to examine whether the dietary protein content of a swine diet can alter UCP2 and UCP3 mRNA abundance and the potential mechanisms for any change in UCP expression through analysis of PPARα, acyl CoA oxidase and PPARγ.

2. Materials and methods

Sixteen crossbred (Yorkshire × Landrace) castrated male pigs (barrows) were maintained on an 18% crude protein (CP) diet until the beginning of the experiment (Table 1). At 55 kg body weight eight of the barrows were switched to a diet containing 12% CP (Table 1) while the other eight pigs remained on the 18% CP diet (control). Power analysis using SigmaStat software (SPSS Science, Chicago, IL) determined that we required 8 pigs per group to have a probability greater than 90% (0.916) of detecting differences at an α = 0.05. Animals were presented with 130% calculated metabolizable energy requirement daily at 0800 h. Animals were maintained on the control diet or the low protein diet from 55 kg body mass until 110 kg body mass. A blood sample was drawn the day prior to euthanasia at approximately 1400 h. Animals were euthanized at 0800 by electrical stunning and exsanguination according to procedures approved by the Institutional Area Animal Use and Care Committee.

Carcass composition was determined using duel energy X-ray absorptiometry (DEXA) on the right side of the carcass according to procedures previously described (Mitchell et al., 1998). Briefly, head and viscera were removed at slaughter and the carcass was split at the midline. The hair and feet remained on the carcass. The right half of each carcass was chilled for 24 h, weighed, standard carcass measurements taken, and then scanned using a Lunar (Madison, WI) DPX-L densitometer.

Tissue samples were collected from the left side of the carcass. Dorsal subcutaneous adipose tissue samples were collected from between the second and fourth thoracic vertebrae, and subsequently, outer (OSQ) and middle (MSQ) layers were separated, diced and frozen in liquid nitrogen. In addition, samples of liver, leaf (perirenal) fat, longissimus muscle (LM), the inner slow-twitch fiber enriched red portion of the semitendinosus (STR) and the outer fast-twitch fiber enriched white portion of the ST (STW) were collected, diced and frozen in liquid nitrogen. Outer and middle subcutaneous adipose tissues were separated for analysis because of their known differences in metabolic activity, while leaf fat has a different metabolic profile from either subcutaneous adipose tissue (Anderson et al., 1972; Rule et al., 1989; Budd et al., 1994). The LM was selected as it represents a large, economically important muscle. The STR and STW were selected because they permit comparison of the potential role of fiber types in the metabolic activity of skeletal muscle (Spurlock et al., 2001).

2.1. Hormone and metabolite analysis

Blood samples were centrifuged at 600 ×g and serum samples were collected with individual Pasteur pipettes. Serum samples were stored at −70 °C for later analyses of hormones and metabolites. Serum insulin was measured using a homologous RIA kit using human standards (Linco Research Inc., St. Charles, MO). Intraassay CV for insulin was 5.4%, while the interassay CV was 9.4%. Serum IGF-1 was acid-ethanol extracted to remove binding proteins and then assayed using a heterologous immunoradiometric kit (Diagnostics Systems Laboratory Inc., Webster, TX) which was previously validated for swine (Balaji et al., 2000). Intraassay CV for IGF-1 was 6.3% while the interassay CV was 4.8%. Serum growth hormone was determined using a homologous RIA kit (Linco Research Inc., St. Charles, MO). Intraassay CV for growth hormone was 11.1%, while the interassay CV was 6.6%. Serum glucose

Table 1
Diet composition

Ingredient	12% Protein [a]	18% Protein [b]
	% of Total	
Corn	78.4	62.5
Soybean meal	9.00	26.1
Whey	6.00	3.75
Soybean oil	2.00	2.00
Lysine hydrochloride	0.17	0.33
Dicalcium phosphate	3.00	3.79
Calcium carbonate	0.50	0.63
Iodized Salt	0.50	0.50
Mineral premix [c]	0.20	0.20
Vitamin mix [c]	0.20	0.20
Selenium premix [d]	0.05	0.05

[a] Calculated nutrient composition: 12% CP; 0.7% lysine; 3.4 Mcal of DE/kg.
[b] Calculated nutrient composition: 18% CP; 1.2% lysine; 3.1 Mcal of DE/kg.
[c] For actual composition of mineral and vitamin premixes, see Campbell et al. (1988).
[d] Provided 22 μg of selenium/kg diet.

(Thermo DMA, Louisville, CO), triglycerides (DMA, Arlington, TX) and non-esterified fatty acids (NEFA, Wako Chemical Co., Richmond, VA) were determined with kit based assays. Serum urea nitrogen was quantified colorimetrically based on a diacetyl monoxime reaction assay described by the World Health Organization (World Health Organization, 2006). Absorbance was determined at 540 nm with the usage of a standard curve (NERL 2336; NERL Diagnostics, East Providence, RI, USA).

2.2. Real-time PCR analysis of gene expression

The genes selected for analysis included porcine UCP2, UCP3, peroxisome proliferator activated receptor α (PPARα), PPARγ and acyl CoA oxidase (ACOX). The uncoupling proteins were selected as described in the Introduction. The PPARs were analyzed because of their role in the regulation of UCP gene expression (Lentes et al., 1999; Sbraccia et al., 2002) in an attempt to identify a mechanism for changes in UCP mRNA abundance. The ACOX was analyzed due to its known role in the peroxisomal oxidation of fatty acids in response to metabolic shifts associated with UCP activity as well as its direct regulation by PPARα (Tugwood et al., 1992; Baillie et al., 1999; Vazquez et al., 2001).

Total RNA was isolated using TRI Reagent according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). Integrity of RNA was assessed via agarose gel electrophoresis and RNA concentration was determined spectrophotometrically using A260 and A280 measurements.

The following primers were used for generating 210-base-pair PCR products corresponding to a portion of the pig UCP2 coding sequence: 5′-CTGCAGATCCAGGGAGAAAG-3′ (forward), 5′-GCTTGACGGAGTCGTAGAGG-3′ (reverse). The primers for UCP3 were used to generate a 200 base-pair product: 5′-ACGATGGATGCCTACAGGAC-3′ (forward), 5′-TCCG-AAGGCAGAGACAAAGT-3′ (reverse). The PPARα primers were used to generate a 196 base-pair product: 5′-GGGATCA-GATGGATCCGTTA-3′ (forward), 5′-AAAGAAGCCCTTG-CAACCTT-3′ (reverse). The PPARγ primers produced a 210 base-pair product: 5′-GCCCTTCACCACTGTTGATT-3′ (forward), 5′-GAGTTGGAAGGCTCTTCGTG-3′ (reverse), which encompasses both PPARγ1 and PPARγ2. The ACOX primers were used to generate a 214 base-pair product: 5′-CTCGCA-GACCCAGATGAAAT-3′ (forward), 5′-AGCCTCGAAGAT-GAGTTCCA-3′ (reverse). All primer sets were designed to span an intron. The UCP2, UCP3, PPARα, PPARγ and ACOX amplicons were excised from an agarose gel, re-amplified, and run through a GenElute PCR clean-up kit (Sigma-Aldrich, St. Louis, MO). The amplicons were subsequently sequenced to confirm identity using automated fluorescent DNA sequencing (ABI 310, Perkin Elmer Applied Biosystems, Foster City, CA). The primers for 18S ribosomal RNA were purchased (QuantumRNA™ Universal 18S Internal Standard; Ambion, Inc; Austin, TX).

Thermal cycling and data acquisition were performed with a Biorad iCycler IQ system (Bio-rad Laboratories Inc., Hercules, CA). Reverse transcription (RT) and real time PCR analysis were performed in a two tube assay. Reverse transcription (RT) was done using Invitrogen's Superscript First-Strand Synthesis System for RT-PCR kit. Master mix was made containing random hexamers (50 ng/μL), 10 mM dNTP mix, RNase-free H_2O, and RNA (1 μg/μL). The RNA mix was annealed at 65 °C for 5 min. A second master mix was prepared of 10X RT buffer, 25 mM $MgCl_2$, 0.1 mM dithiothreitol, and 1.0 μL RNaseOut. This second master mix was added to the RNA mix and incubated at 25 °C for 2 min. Superscript II was then added and incubated at 25 °C for 10 min, 42 °C for 50 min, 70 °C for 15 min. An aliquot of RNase H (1.0 μL) was added and incubated at 37 °C for 20 min.

Real time PCR was done using the IQ sybr green supermix kit (Biorad, Hercules, CA). A 24 μL reaction mix was made containing 12.5 μL sybr green supermix, 1.0 μL forward primer (10 μM), 1.0 μL reverse primer (10 μM) and 9.5 μL sterile water. This reaction mix was added to each well, followed by 1.0 μL reverse transcription product (25 μL total volume).

Parameters for UCP2 and UCP3 PCR were as follows: 1 cycle 95 °C for 15 min (PCR activation), followed by 30 cycles, 94 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 8 min. Parameters for PPARα, PPARγ and ACOX were as follows: 1 cycle 95 °C for 15 min (PCR activation), followed by 30 cycles, 94 °C for 15 s, 57 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 8 min. Melting curve analysis was performed on all real time PCR reactions to confirm specificity and identity of the real time PCR products. Specificity of real time PCR products was further confirmed by agarose gel electrophoresis. The two-step real time PCR for UCP2, UCP3, PPARα, PPARγ, ACOX and 18 S were optimized for linearity (exponential amplification) from >20 to <30 cycles under the conditions described above.

2.3. Quantification of gene expression

At the end of the PCR, baseline and threshold crossing values (C_T) for UCP2, UCP3, PPARα, PPARγ, ACOX and 18 S were calculated using the Opticon Monitor Software (Version 1.06; MJ Research, Waltham, MA) and the C_T values were exported to Microsoft Excel for analysis. The relative expression of UCP2, UCP3, PPARα, PPARγ, ACOX mRNA and 18 S rRNA were calculated using the comparative C_T method according to manufacturer's literature (MJ Research). The C_T values were transformed to their respective antilogarithmic values. The relative amount of UCP2, UCP3, PPARα, PPARγ and ACOX mRNA, standardized against the amount of 18 S rRNA was expressed as $\Delta C_T = [C_T UCP - C_T 18S]$. Values are presented as the mean ± SEM of determinations from the tissues of eight individual animals per dietary treatment group.

2.4. Statistical analysis

Data were analyzed by one-way analysis of variance using SigmaStat software (SPSS Science, Chicago, IL) to test for treatment effects. Mean separation was analyzed using Student–Newman–Keuls test. Means were defined as significantly different at $P < 0.05$.

3. Results

There was no difference in the initial body weights and final body weights between the two dietary treatment groups ($P > 0.05$; Table 2). Average daily gain and the days on trial did not differ between animals fed the 12% CP diet and the animals fed the 18% CP diet ($P > 0.05$). Feed intake measurements could not be made due to facility considerations.

Body composition was not affected by feeding a 12% CP diet from 55 kg until 110 kg body weight relative to the 18% CP diet (Table 3). Half carcass weight was not altered by dietary protein content. Carcass lean, carcass fat and bone mineral content (BMC) in the half carcass were not altered by dietary protein content as analyzed with DEXA ($P > 0.05$). In addition, longissimus muscle area and subcutaneous adipose tissue thickness (10th rib back fat) were not affected by dietary protein content ($P > 0.05$).

Serum GH was depressed 43% by feeding a 12% CP diet relative to an 18% CP diet ($P < 0.05$; Table 4). Serum insulin and IGF-I were not affected by dietary protein concentration ($P > 0.05$). Serum glucose was not altered by protein content of the diet ($P > 0.05$). However, non-esterified fatty acids (NEFA) and triglycerides were reduced by 28% and 40%, respectively; with the feeding of a 12% CP diet relative to an 18% CP diet ($P < 0.05$). Serum urea nitrogen was reduced by 30% with the feeding of a 12% protein diet ($P < 0.001$).

Feeding a diet containing 12% CP reduced UCP2 mRNA abundance in MSQ by 56% ($P < 0.001$), in OSQ by 35% ($P < 0.001$), in LM by 19% ($P < 0.05$; Fig. 1), and in STR by 24% ($P < 0.05$). The UCP2 mRNA abundance was not altered by dietary protein content in the liver or leaf fat ($P > 0.05$).

The mRNA abundance of UCP3 was 38% lower in MSQ ($P < 0.01$; Fig. 2) and 29% lower in STW ($P < 0.01$) of pigs fed a diet containing 12% CP than in pigs fed 18% CP. The UCP3 mRNA did not differ in the leaf fat, OSQ, LM, or STR of pigs fed 12% CP and 18% CP ($P > 0.05$). Since neither UCP2 nor UCP3 mRNA abundance were altered by dietary protein in the liver or leaf fat, further analysis was not performed on those two tissues.

The concentration of dietary protein affected PPARα mRNA abundance in several tissues of the pig (Fig. 3). The PPARα mRNA abundance was 53% lower in MSQ ($P < 0.001$) and 26% lower in STR ($P < 0.01$) of pigs fed 12% CP than in pigs fed 18% CP. The PPARα mRNA abundance in OSQ, LM and STW was not affected by dietary protein ($P > 0.05$).

The mRNA abundance of PPARγ was influenced by dietary protein in the MSQ and STR (Fig. 4). The PPARγ mRNA abundance of MSQ was reduced by 37% with the feeding of 12% CP ($P < 0.001$), relative to feeding an 18% CP diet. Abundance of PPARγ was reduced by 22% in STR with the feeding of 12% CP relative to 18% CP ($P < 0.05$).

The ACOX mRNA abundance was 32% lower in MSQ from pigs fed 12% CP than in pigs fed 18% CP ($P < 0.01$; Fig. 5). Similarly, ACOX mRNA abundance was 24% lower in the STR following the feeding of 12% CP than 18% CP ($P < 0.05$). The concentration of dietary protein did not affect ACOX mRNA abundance in OSQ, LM or STW of pigs ($P > 0.05$).

4. Discussion

Expression of UCP2 and UCP3 in the adipose tissues demonstrated tissue specificity as leaf fat did not respond to dietary protein, while MSQ and OSQ UCP2 mRNA abundance were reduced by more than 30% with feeding of a 12% CP diet, and only MSQ UCP3 mRNA abundance was affected by dietary protein content. Masanés et al. (2002) have previously demonstrated that subcutaneous adipose tissue UCPs are more responsive to dietary protein content than internal sites of deposition. The relative difference in gene expression or difference in gene regulation among various sites of adipose tissue deposition has not been thoroughly examined in the pig,

Table 3
Carcass measurements from pigs fed diets containing 12% CP or 18% CP

Item	Treatment group			
	12% CP	SE 18%	CP	SE
Carcass mass, kg	81.9	0.8	82.5	0.9
Half carcass mass, kg	40.8	0.4	41.3	0.4
Carcass length, cm	80.2	0.6	80.4	0.8
10th rib fat, cm	3.14	0.26	3.21	0.13
Longissimus muscle area, cm^2	27.32	1.17	27.11	1.69
Tissue fat, %	32.78	1.67	33.91	1.08
Tissue fat, kg	12.98	0.66	13.59	0.43
Tissue lean, %	67.22	1.64	66.09	0.62
Tissue lean, kg	26.63	0.65	26.48	0.25
BMC, %[a]	2.25	0.09	2.25	0.06
BMC, g	891.4	34.8	902.7	22.3

No effects of diet ($P > 0.05$), $n = 8$.
[a] BMC = bone mineral content.

Table 2
Effects of dietary protein concentration on the growth performance of pigs

Item	Treatment group			
	12% CP	SE	18% CP	SE
Initial wt, kg	49.6	0.5	51.5	0.8
Final wt, kg	110.7	0.6	110.3	1.4
Days on trial	56.4	1.6	59.8	1.6
ADG, g	1088	30	989	37

No diet effect ($P > 0.05$).

Table 4
Effects of dietary protein concentration on selected serum hormones and metabolites in pigs

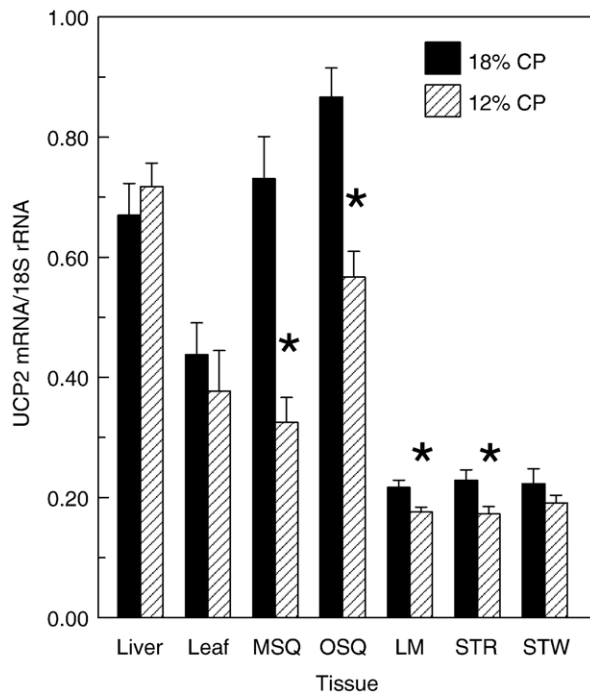| Item	Treatment group			
	12% CP	SE	18% CP	SE
IGF-I, ng/mL	469	25	445	28
Insulin, μU/mL	22.7	3.8	18.9	3.7
Growth Hormone, ng/mL	1.6 *	0.1	2.3	0.3
Glucose, mMol/L	5.23	0.25	4.87	0.31
Triglycerides, mg/L	28 *	4	46	7
NEFA, uEq/L	99.4 *	7.6	137.2	11.1
Serum Urea Nitrogen, mg/L	262 *	12	372	15

* Diet effect ($P < 0.05$), $n = 8$.

Fig. 1. Relative uncoupling protein 2 (UCP2) mRNA abundance in tissues from barrows at 110 kg body mass after feeding 18% crude protein (18% CP) or 12% crude protein (12% CP) ad libitum from approximately 50 kg body mass; based on extraction of total RNA and subsequent real-time PCR analysis for UCP2 mRNA abundance. Data are expressed as the mean ratio±SEM of specific UCP2 mRNA: 18S rRNA for eight pigs in each group. The asterisks indicate that the means differ from that of pigs fed 18% CP (P<0.05, n=8). Leaf = leaf fat; MSQ = middle subcutaneous adipose tissue; OSQ = outer subcutaneous adipose tissue; LM = longissimus; STR = semitendinosus, red portion; STW = semitendinosus, white portion.

but the present data indicate unique regulation of the uncoupling proteins among the three sites of deposition. For example, the metabolic and physiological adaptations to differences in dietary protein content altered the mRNA abundance of PPARα, PPARγ and ACOX in the MSQ, parallel to changes in UCP2 and UCP3 mRNA abundance. Yet in the OSQ, only UCP2 was affected by the dietary protein content, none of these other genes were affected.

A previous study has demonstrated that low protein and high protein diets both reduce UCP3 mRNA abundance in subcutaneous adipose tissue of obese rats (Masanés et al., 2002). The present study has confirmed this response by demonstrating that feeding a 12% CP diet to swine results in lower UCP3 mRNA abundance in MSQ than when feeding an 18% CP diet.

Reductions in PPARα and PPARγ were observed with the feeding of a 12% CP diet. The endogenous ligands for both of these PPARs appear to be fatty acids and/or their metabolites (Nakamura et al., 2004; Jump et al., 2005). Serum NEFA has been specifically demonstrated to alter PPARγ expression in muscle (Sbraccia et al., 2002). Therefore, the reduction in serum NEFA in the present study may have contributed to the reductions in PPARα and PPARγ. However, no significant correlation was detected between serum NEFA and the PPARs. This may suggest the overall metabolic shifts within the tissue

due to low protein feeding may contribute to the changes in PPAR expression. Both UCP2 (Lentes et al., 1999) and UCP3 (Acin et al., 1999) contain PPAR response elements in their promoters, thereby regulating the transcription of these UCPs. Thus, the reduction in the PPARs in the present study may have contributed to the observed reduction in UCP2 and UCP3 mRNA abundance in the MSQ with the feeding of 12% CP relative to 18% CP. Pearson correlation analysis demonstrated significant correlations between UPC2 and PPARα (r=0.936, P<0.0001) or PPARγ (r=0.920, P<0.0001). The parallel reduction in ACOX in the MSQ (r=0.906, P<0.0001) reflects the regulatory role that PPARα has for this enzyme, since the ACOX gene also contains a PPAR response element (Tugwood et al., 1992). Secondly, ACOX has been demonstrated to induce peroxisomal fatty acid oxidation in adipose tissue in response to PPARα activation (Vazquez et al., 2001). As UCP2 is now believed to function in peroxisomal fatty acid oxidation (Jezek et al., 2004), the reduction in mRNA abundance for UCP2 and ACOX would indicate that peroxisomal fatty acid metabolism is reduced with feeding a low protein diet in swine.

In contrast to adipose tissue, soleus muscle UCP3 was elevated with the feeding of either a low or high protein diet to obese rats, but reduced in the soleus of lean rats (Masanés et al., 2002). Masanés et al. (2002) demonstrated a muscle specific dietary protein response in UCP3 expression in lean rats with
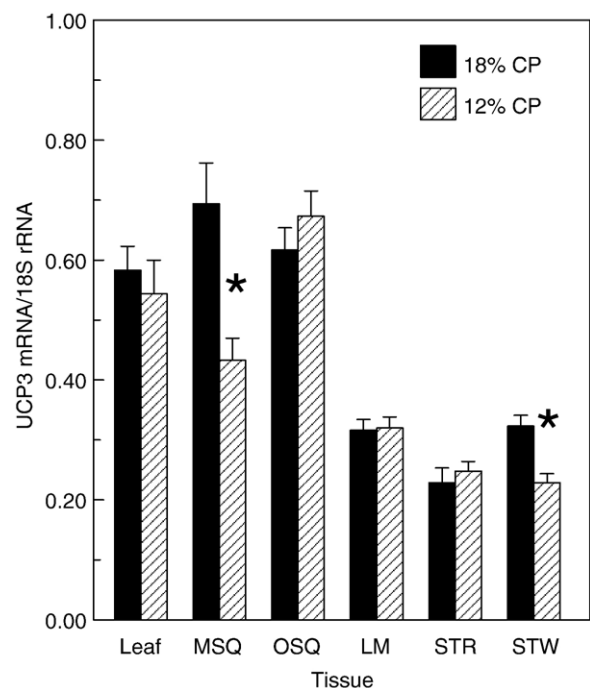

Fig. 2. Relative uncoupling protein 3 (UCP3) mRNA abundance in tissues from barrows at 110 kg body mass after feeding 18% crude protein (18% CP) or 12% crude protein (12% CP) ad libitum from approximately 50 kg body mass; based on extraction of total RNA and subsequent real-time PCR analysis for UCP3 mRNA abundance. Data are expressed as the mean ratio±SEM of specific UCP3 mRNA: 18S rRNA for eight pigs in each group. The asterisks indicate that the means differ from that of pigs fed 18% CP (P<0.05, n=8). Leaf = leaf fat; MSQ = middle subcutaneous adipose tissue; OSQ = outer subcutaneous adipose tissue; LM = longissimus; STR =semitendinosus, red portion; STW = semitendinosus, white portion.
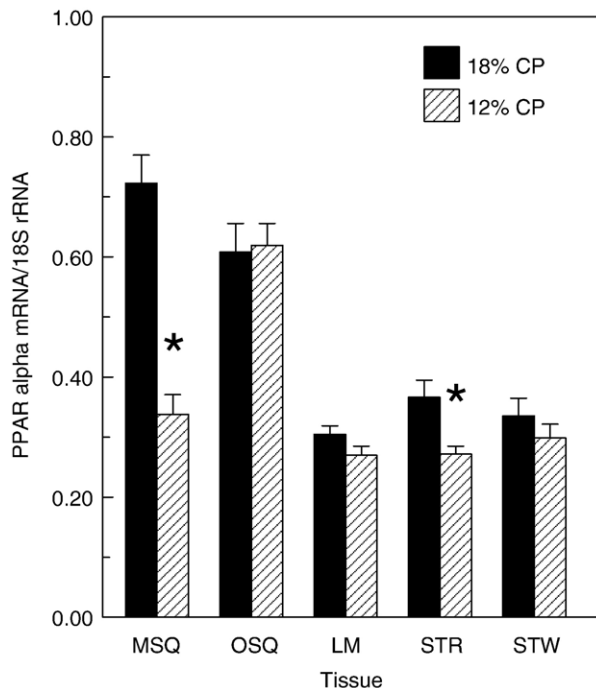
Fig. 3. Relative peroxisome proliferator activated receptor α (PPARα) mRNA abundance in tissues from barrows at 110 kg body mass after feeding 18% crude protein (18% CP) or 12% crude protein (12% CP) ad libitum from approximately 50 kg body mass; based on extraction of total RNA and subsequent real-time PCR analysis for PPARα mRNA abundance. Data are expressed as the mean ratio ± SEM of specific PPARα mRNA: 18 S rRNA for eight pigs in each group. The asterisks indicate that the means differ from that of pigs fed 18% CP ($P<0.05$, $n=8$). MSQ = middle subcutaneous adipose tissue; OSQ = outer subcutaneous adipose tissue; LM = longissimus; STR = semitendinosus, red portion; STW = semitendinosus, white portion.

the slow twitch-oxidative soleus responding to dietary protein and the fast twitch-glycolytic tibialis anterior not responding. However, Petzke et al. (2005) reported no effect of dietary protein on UCP3 mRNA abundance in the biceps femoris containing mixed fiber types. The present study also demonstrated muscle specific responses to dietary protein with UCP3 mRNA abundance altered only in STW, the portion of the semitendinosus enriched with glycolytic fibers, relative to the STR that is enriched with more oxidative fibers and the LM which contains more of a mixture of both fiber types. This change in UCP3 was not associated with any changes in PPARα or PPARγ. Samec et al. (2002) have previously reported that fast twitch glycolytic fibers do not appear to require changes in the expression of PPARγ to modulate UCP2 or UCP3 expression.

A previous study in neonatal swine (Mostyn et al., 2005) demonstrated that UCP3 mRNA abundance was higher in neonatal skeletal muscle than adipose tissue, in contrast to the present study wherein relative expression appeared higher in adipose than skeletal muscle. The data in both studies was expressed relative to 18S rRNA. As 18S rRNA levels change with age (Mori et al., 1978; Miller et al., 1980), comparison of gene expression in neonatal animals with market weight animals must be performed with caution, but may account for this apparent age effect.

The PPARγ primers used for real-time PCR in the present study measured both PPARγ1 and PPARγ2. The predominate PPAR in human skeletal muscle is PPARγ1 (Kruszynska et al., 1998) while its expression in pig muscle is unknown. PPARγ2 is found almost exclusively in pig adipose tissue (Houseknecht et al., 1998). Grindflek et al. (1998) were able to detect only limited PPARγ (1 and 2) mRNA in skeletal muscle (undefined) by Northern analysis, while Houseknecht et al. (1998) were unable to detect any PPARγ (1 and 2) mRNA in longissimus muscle by Northern or RNase protection analysis, or PPARγ protein by Western analysis. The level of PPARγ expression in swine muscle is a fraction of that in adipose (Grindflek et al., 1998). It has been proposed that any expression of PPARγ1 or 2 specific to muscle cells could be masked by expression of PPARγ2 from adipocytes present in the muscle tissue. Reducing dietary protein by 20% has been demonstrated to double lipid content of longissimus in restriction fed animals (da Costa et al., 2004); while feeding 75% of dietary protein requirement increased intramuscular fat content by 40% in the longissimus. The increase in lipid content was the consequence of hypertrophy of intra and intermuscular adipocytes and not hyperplasia (Gondret and Lebret, 2002). Vidal-Puig et al. (1997) and Kruszynska et al. (1998) have demonstrated that adipocyte contamination of skeletal muscle makes a very limited contribution to overall skeletal muscle PPARγ mRNA
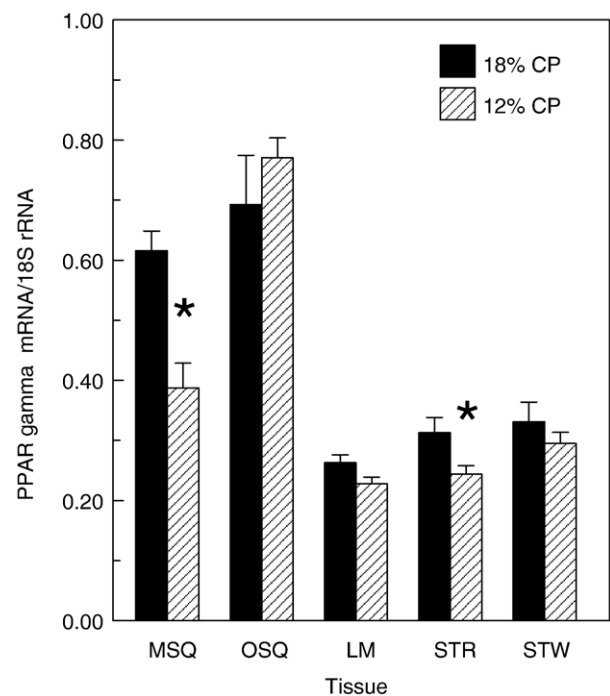

Fig. 4. Relative peroxisome proliferator activated receptor γ (PPARγ) mRNA abundance in tissues from barrows at 110 kg body mass after feeding 18% crude protein (18% CP) or 12% crude protein (12% CP) ad libitum from approximately 50 kg body mass; based on extraction of total RNA and subsequent real-time PCR analysis for PPARγ mRNA abundance. Data are expressed as the mean ratio ± SEM of specific PPARγ mRNA: 18S rRNA for eight pigs in each group. The asterisks indicate that the means differ from that of pigs fed 18% CP ($P<0.05$, $n=8$). MSQ = middle subcutaneous adipose tissue; OSQ = outer subcutaneous adipose tissue; LM = longissimus; STR = semitendinosus, red portion; STW = semitendinosus, white portion.
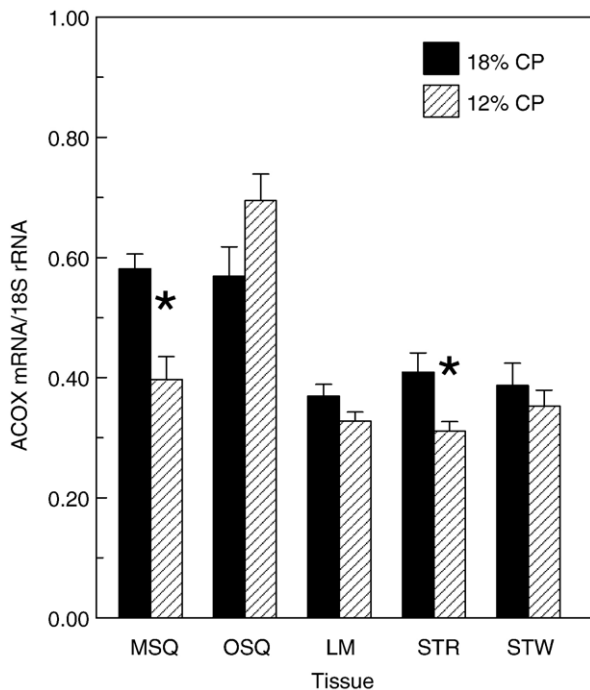
Fig. 5. Relative acyl CoA oxidase (ACOX) mRNA abundance in tissues from barrows at 110 kg body mass after feeding 18% crude protein (18% CP) or 12% crude protein (12% CP) ad libitum from approximately 50 kg body mass; based on extraction of total RNA and subsequent real-time PCR analysis for ACOX mRNA abundance. Data are expressed as the mean ratio±SEM of specific ACOX mRNA: 18 S rRNA for eight pigs in each group. The asterisks indicate that the means differ from that of pigs fed 18% CP ($P<0.05$, $n=8$). MSQ = middle subcutaneous adipose tissue; OSQ = outer subcutaneous adipose tissue; LM = longissimus; STR = semitendinosus, red portion; STW = semitendinosus, white portion.

abundance in humans, but it cannot be excluded from making a significant contribution to PPARγ expression in porcine skeletal muscle.

Uncoupling protein 2 mRNA abundance was altered in the LM and STR with dietary protein, but not in the STW. The reduction in UCP2 mRNA in the STR abundance with feeding of 12% CP appeared to be associated with a reduction in both PPARα ($r=0.948$, $P<0.0001$) and PPARγ ($r=0.949$, $P<0.0001$) and a subsequent reduction in ACOX ($r=0.943$, $P<0.0001$). Changes in LM UCP2 mRNA abundance were not associated with any changes in PPARα or PPARγ, as was the case for STW UCP3. The LM of swine contains a mixture of fibers, but primarily glycolytic fibers (Solomon et al., 1990). Thus, these muscle specific effects would indicate that red oxidative muscle fibers may be more associated with fatty acid metabolism than glycolytic fibers, as previously demonstrated (Ponsot et al., 2005).

Petzke et al. (2005) have demonstrated that feeding a low, but adequate protein diet (14% CP) to rats reduced hepatic UCP2 mRNA abundance by approximately 26% relative to a 26% CP diet. No change in hepatic UCP2 mRNA abundance was detected in the present study with feeding a 12% CP diet relative to an 18% CP diet, which may indicate that the difference in protein content of the diets was inadequate to detect an effect. Uncoupling protein 3 was not quantified for

liver as it is not expressed as previously reported for swine (Damon et al., 2000; Ramsay and Richards, 2007) and rats (Lanni et al., 2002).

No antibodies exist for quantifiable UCP2 or UCP3 protein measurements. The only effective UCP2 antibody (Mostyn et al., 2004) has recently been shown to not cross react well with pig UCP2 (Mostyn et al., 2005). No UCP3 antibody has been shown to cross-react effectively with pig UCP3 (Mostyn et al., 2004). This makes it quite difficult to determine the potential relationship between UCP mRNA abundance and UCP protein levels in the pig. Previous studies have demonstrated that UCP2 and UCP3 mRNA and protein levels do not necessarily correlate (Mostyn et al., 2004; Pecqueur et al., 2001). Thus, caution should be used in interpreting the relationship between changes in porcine UCP mRNA abundance and potential UCP activity.

This study was not designed as a performance trial to compare the relative benefits or deficiencies of a 12% CP diet relative to an 18% CP diet. However, the present study found that the feeding a 12% CP diet relative to an 18% CP diet did not affect weight gain as measured by average daily gain. Previous studies in market weight swine have demonstrated that low protein diets (12–13% CP) reduce (Mitchell et al., 1991; Cromwell et al., 1993; Ivers and Veum, 1993) or have no effect on weight gain (Adeola et al., 1990; Knowles et al., 1998; Kerr et al., 2003).

The absence of any changes in carcass composition was not entirely unexpected. Several previous studies have demonstrated that feeding a diet containing 12–13% CP have not detected a dietary protein effect on fat and lean accumulation or longissimus muscle area (Adeola et al., 1990; Mitchell et al., 1991; Knowles et al., 1998); others have reported that a 12–13% CP diet reduces lean accumulation and longissimus muscle area (Adeola and Young, 1989; Cromwell et al., 1993; Kerr et al., 1995). In general, an impact of low dietary protein on carcass composition is most apparent when the low protein diet is introduced at a younger age. As animals were exposed to the 12% CP diet at approximately 55 kg in the present study, they may have been beyond an age/weight to have a major impact on composition.

The present study detected lower serum GH concentration in market weight pigs fed 12% CP than 18% CP. Previous studies have either reported no change in serum GH (Campbell et al., 1990; Caperna et al., 1990) or an increase in serum GH (Atinmo et al., 1976a) with manipulation of dietary protein content in younger pigs. An elevation in serum GH is apparent with extreme protein calorie malnutrition (Atinmo et al., 1976a; Breier, 1999), however this was not the case in the present study as the protein concentration was 12% in the low protein diet.

Atinmo et al. (1978) reported that feeding an 8% CP diet to young pigs for eight weeks produced lower serum glucose and higher NEFA than in grower pigs fed 18% CP. Gomez et al. (2002) found that feeding 10–12% CP diets relative to 14–16% CP to 30 to 60 kg pigs increased serum glucose and NEFA. The present study did not detect a difference in serum glucose between dietary protein groups which agrees with the reports of

several other labs (Seve et al., 1993; Brameld et al., 1996), while feeding a 12% CP diet reduced serum NEFA and triglycerides in 110 kg swine, in agreement with Matthews et al. (1998). A reduction in serum GH could contribute to a reduction in serum NEFA, as GH is a major lipolytic hormone that elevates NEFA in swine (Dunshea et al., 1992). However the very small change in serum GH, without a change in IGF-I, raises the question of whether or not GH has an impact on NEFA concentration.

The present study did not observe a difference in serum IGF-I between market weight swine fed 12% or 18% CP between 50 and 110 kg body mass; in agreement with other studies that have found no difference in serum IGF-I with manipulation of dietary protein (Seve et al., 1993; Brameld et al., 1996; Farmer et al., 2004). Caperna et al. (1990) and Atinmo et al. (1976b) reported that feeding low protein diets to young pigs produces lower serum insulin concentrations than high protein diets. However, no differences in serum insulin were detected in the larger, older pigs fed 12% versus 18% CP in the present study. Brameld et al. (1996) and Seve et al. (1993) also reported no effect of dietary protein on serum insulin.

A reduction in serum urea nitrogen is a common characteristic of feeding a low protein diet when supplemented with various crystalline amino acids to swine (Gomez et al., 2002; Knowles et al., 1998; Seve et al., 1993; Kerr and Easter, 1995; Figueroa et al., 2002; Figueroa et al., 2003). This was corroborated by the present study.

In summary, the data from the present study would suggest that the metabolic changes associated with feeding of the 12% CP diet, relative to the 18% CP diet, resulted in a reduction in PPARα and PPARγ activity, and consequently a reduction in UCP2 and UCP3 mRNA abundance. Both UCP2 and UCP3 demonstrate much weaker uncoupling activity than UCP1 (Esteves and Brand, 2005), and through a number of studies have been shown to have limited roles in uncoupling activity and heat production (Jezek and Garlid, 1998; Erlanson-Albertsson, 2003; Schrauwen et al., 2006). Recent studies have associated UCP3 with the intracellular transport of fatty acid anions from the mitochondrial matrix to the cytosol (Himms-Hagen and Harper, 2001), but also with reducing reactive oxygen species in skeletal muscle (MacLellan et al., 2005). Uncoupling protein 2 has been associated with lipoperoxidation and with reducing reactive oxygen species that are generated by mitochondria with metabolic activity (Negre-Salvayre et al., 1997; Jezek et al., 2004). Thus the data from the present study would suggest that the parallel reduction in ACOX mRNA and UCP2 mRNA abundance in OSQ and STR in response to a low protein diet may affect lipoperoxidation rates and production of reactive oxygen species, but this requires further investigation.

Acknowledgements

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